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RESEARCH ARTICLE

Association between saliva PSA and serum PSA in conditions with prostate adenocarcinoma

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Abstract

In recent years, saliva samples have attracted attention as specimens, which may be used for cancer diagnosis. Prostate-specific antigen (PSA) is the most useful tumor marker for prostate adenocarcinoma (PA). We examined whether there is an association between saliva PSA and serum PSA in patients with PA using enzyme-linked immunosorbent assay. Human subjects were classified into two groups: a low-serum PSA concentration group ($n = 20$) (<2.5 ng/mL) and a high-serum PSA concentration group with high risk of recurrence or metastasis ($n = 11$) (≤2.5 ng/mL). There were significant differences in saliva PSA concentration between these groups ($p < 0.05$). Saliva PSA concentration correlated very well with serum PSA concentration in the high-serum PSA concentration group ($\gamma = 0.910$, $p < 0.001$) using Spearman's rank test, but no correlation in the low-serum PSA concentration group. This result suggests that saliva PSA is associated with blood PSA in patients with recurrent or metastatic PA and may, therefore, be a useful PA biomarker.

Keywords: ELISA; LNcaP; saliva test; salivary gland

Introduction

The salivary gland is an exocrine gland composed of acinar and ductal cells. Various growth factors, as well as various digestive enzymes, and antibacterial factors are produced in the salivary gland, and these salivary products have been associated with the maintenance of oral and systemic health (Saruta et al. 2010). Interestingly, saliva may include many components that are derived from blood because acinar cells produce saliva using blood materials (plasma). For instance, the concentration of measles-specific IgM antibody in saliva samples is in good agreement with that in serum samples (Mokhtari et al. 2003). Measles-specific IgM antibody is not produced in the salivary gland. Clearly, if a saliva test is used to measure blood component levels, then production of these blood components by salivary glands is not desirable.

Saliva can be collected at lower cost and with smaller burden on patients compared with the collection of blood. In addition, saliva collection can be conducted in any environment and requires no special skills or equipment and may, therefore, be particularly useful in countries where blood collection is difficult. Since the collection of saliva is a noninvasive procedure, identification of biomarkers in saliva could lead to the establishment of a nonpainful examination, which is the desire of all patients. Saliva is, therefore, considered a better medical laboratory sample than blood or urine. Till date, saliva-based cancer diagnosis has been examined for oral, breast, and pancreatic cancer using metabolomics, proteomics, or DNA/RNA arrays (Sugimoto et al., 2010, Brooks et al., 2008, Streckfus et al., 2008). Therefore, great attention is being paid

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to the potential of saliva for clinical application in the diagnosis of a variety of diseases, including cancer. However, there is no development of saliva tests in use for the detection of various primary cancers or of their recurrence.

Prostate-specific antigen (PSA) is an androgen-regulated serine protease that is a member of the tissue kallikrein family of proteases (Rittenhouse et al., 1998). PSA is primarily produced by prostate ductal and acinar epithelium and is secreted into the lumen. PSA in the blood occurs in three forms: free PSA, PSA complexed with α1-antichymotrypsin, and PSA complexed with β2-macroglobulin. A serum PSA test has been widely regarded as the most useful clinical marker for the detection, screening, staging, prognosis, and monitoring of therapy in prostate adenocarcinoma (PA) (Mistry & Cable, 2003). Measurement of the serum PSA level has become particularly important for achieving efficient detection of clinically significant cancer in the first step of mass screening.

Ayatollahi et al. reported that a saliva sample can be used instead of a serum sample for estimation of free-total PSA and total PSA-protein levels in men without prostate disease (Ayatollahi et al., 2007). Therefore, the purpose of this investigation was to determine whether there is an association between saliva PSA and serum PSA in patients with PA using a conventional PSA-enzyme-linked immunosorbent assay detection system.

Methods

Animals

Nine-week-old male CB17-scid/scid mice (severe combined immunodeficiency [SCID] mice; CLEA Japan, Tokyo, Japan) were used for this study. Mice were housed in groups of four animals per cage in a pathogen-free environment and were provided with sterile water and food. The experimental protocol used in this study was reviewed and approved by the Committee of Ethics on Animal Experiments of Kanagawa Dental College and was carried out in accordance with the Guidelines for Animal Experimentation of Kanagawa Dental College.

Cell culture and tumor cell injection

The human prostate PSA-producing adenocarcinoma cell line, LNCaP, was obtained from Prof. Kubota, Department of Urology, Yokohama University, School of Medicine and was grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA). To carry out a detailed examination of the correlation between serum PSA and saliva PSA, we formed tumor masses of various sizes by resuspension of the cells in phosphate-buffered saline (PBS) to a concentration of 1×10^6 ($n = 2$), 10^7 ($n = 3$), or 10^8 ($n = 2$) cells/mL. The cell suspension (0.1 mL) was mixed with 0.3-mL Matrigel (Becton Dickinson and Company, Franklin Lakes, NJ, USA) in a 1-cc syringe, allowed to warm at room temperature for 5 min with gentle mixing, and then injected under the skin in the back of the SCID mice (total of seven mice) (Wilson et al. 1997). The diameter of the tumor cell mass was measured after 30 days. A group of mice injected with PBS and Matrigel was used as a control ($n = 7$).

Sampling of saliva, serum, and submandibular gland

Mice were killed 30 days after injection of tumor cells. Saliva sampling was performed under mild anesthesia using diluted pentobarbital sodium (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan). Secretion of saliva was stimulated by intraperitoneal injection (0.1 mL) of a cocktail containing 0.2-mg isoproterenol (Santen Pharmaceutical Co., Ltd., Osaka, Japan) and 0.05-mg pilocarpine (Kowa Company, Tokyo, Japan) per 100 g body weight. The doses of isoproterenol and pilocarpine used are optimal for stimulation of saliva secretion (Hu et al. 1992). Five minutes after injection of the drugs, saliva was continuously collected over a 10-min period from the oral cavity using Einmal–Kapillarpipetten ring caps (Hirschmann Laborgerate, GmbH and Co. KG, Eberstadt, Germany). The saliva samples were centrifuged at 2000 rpm for 10 min at 4°C. The cell-free supernatant saliva specimens were stored at –80°C until assayed.

Abdominal section was performed under deep anesthesia after saliva sampling. Serum was prepared from blood collected from mouse hearts and was stored at –80°C.

The submandibular gland was removed and homogenized in complete Lysis-M buffer (pH 7.6) (Roche Diagnostics Ltd., Lewes, UK). The homogenate was centrifuged at 2000 rpm for 10 min, and the supernatant was collected and stored as a frozen sample at –80°C.

PSA assay

PSA concentrations in serum, saliva, and submandibular gland samples were determined using an ultrasensitive chemiluminescent enzyme immunoassay. PSA values were measured using an Access Hybritech PSA kit and the UniCel DxI 800 auto-analyser (Beckman-Coulter, Fullerton, CA, USA) according to the manufacturer's instructions (Slev et al. 2008). The minimum PSA detection level was 0.003 ng/mL.

Human subjects

Serum and saliva concentrations of total PSA were measured in 31 men between 56 and 88 years of age (mean 74.7 years), who presented for routine checkup. The patients were selected from those treated at Iwaki Kyouritsu General Hospital, Division of Urology (Fukushima, Japan) between April 2008 and March 2009. The diagnosis was histologically confirmed as PA based on the World Health Organization classification and the Gleason grading system. We selected patients who were

followed up after hormonal therapy or were undergoing hormonal therapy. Human participants in this study were classified into two groups: a high-serum PSA concentration group ($n=11$) (≥2.5 ng/mL) with high risk of recurrence or metastasis and a low-serum PSA concentration group ($n=20$) (<2.5 ng/mL). A value of 2.5 ng/mL was chosen as the cut-off value (Figure 1). Recurrence or metastasis was clinically identified in all patients in the high-serum PSA concentration group, but not in the low-serum PSA concentration group. The study was approved by the interdepartment ethics committee of Iwaki Kyouritsu General Hospital and included the informed consent of all participants.

Sample collection

All patients were checked by dentists, and patients with significant periodontal disease were excluded. Stimulated saliva was collected using a salivette with citrate-containing cotton (SARSTEDT AG and Co. Numbrecht, Germany). Samples of saliva were centrifuged at 2000 rpm for 10 min. The Bradford assay was performed by means of a Quick Start protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Saliva and serum samples were stored at –80° before examination.

RT-PCR analysis of PSA

Human salivary glands were isolated at neck dissection of oral squamous cell carcinoma. Reverse transcriptase (RT) and polymerase chain reaction (PCR) were performed using a First-Strand cDNA Synthesis kit for RT-PCR (AMV) (Roche) and a ThermoScript RT-PCR kit (TaKaRa Bio Inc., Shiga, Japan) according to the instruction manuals. RT was primed using Random 9 mers and cDNA synthesis was performed at 30°C for 10 min, 42°C for 20 min, 99°C for 5 min, and 5°C for 5 min in one cycle. Oligonucleotide primers were designed to amplify a 289-bp fragment of human PSA DNA. Primer sequences were 5′-CCC ACA CCC GCT CTA CGA TA-3′ (sense) and 5′-ACC TTC TGA GGG TGA ACT TGC G-3′ (antisense) (Ishikawa et al. 1998). The RT product was amplified using Tag DNA polymerase after 10-min denaturation at 95°C, followed by 25 cycles each with denaturation at 94°C for 1 min, primer annealing at 58°C for 2 min, product extension at 72°C for 3 min, and final extension 72°C for 5 min.

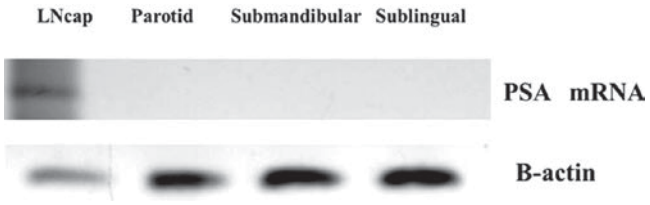

Figure 1. Plot of saliva prostate-specific antigen (PSA) concentration in adenocarcinoma patients. Patients were classified into two groups based on a serum PSA concentration cutoff value of 2.5 ng/mL. The low-serum PSA group shows patients with a PSA concentration of <2.5 ng/mL ($n=20$). The high-serum PSA group shows patients with a PSA concentration of ≥2.5 ng/mL ($n=11$).

Statistical analysis

Data are expressed as means ± SD, and a value of $p<0.05$ was considered statistically significant. A test of significance and the coefficient of correlation were calculated using SPSS Version 17.0 Statistical Software (SPSS, Inc., Lake Oswego, OR, USA). Spearman's correlation coefficient was used to assess the relationship between two groups in the animal or clinical studies. Statistical significance of differences between two groups was determined using Mann–Whitney U-test.

Results

Animal study

We analyzed tissue and serum PSA levels in a mouse model injected with LNCaP PSA-producing cells. The mean tumor size was 1.96 ± 1.57 cm (range, 0.04–4.6 cm) in the LNCaP injection group. Tumor development was not detected in the control group injected with PBS and Matrigel. The mean serum PSA concentration was 57.419 ± 55.401 ng/mL (range, 29.5–145 ng/mL) in the LNCaP injection group. There was no serum PSA in the control group (Table 1). When Spearman's rank test was performed, a significant linear relationship between tumor size and serum PSA concentration was determined in the LNCaP injection group. Tumor size correlated well with serum PSA concentration ($\gamma=0.991$, $p<0.05$) (Figure 2). The mean intra-tissue PSA concentration of the submandibular gland was 0.287 ± 0.213 ng/mL (range 0.107–0.717 ng/mL) in the LNCaP injection group, but no PSA was detected in the control group (Table 1). The intra-tissue PSA concentration was clearly lower than the serum PSA concentration. However, there was a weak correlation between the serum PSA and the intra-tissue PSA concentration of submandibular gland using Spearman's rank test ($\gamma=0.393$, $p<0.05$) (Figure 3). Therefore, the intra-tissue PSA concentration

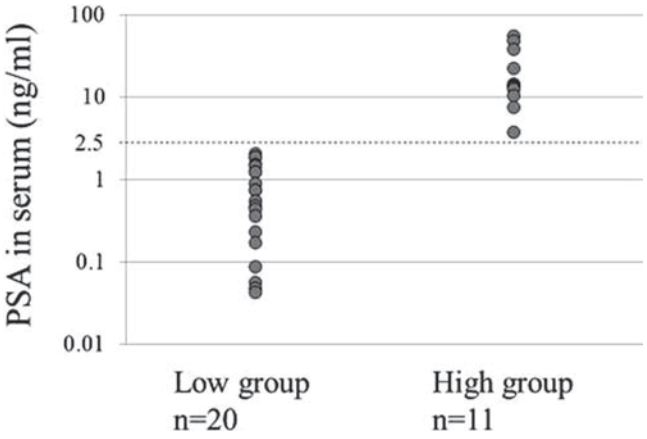

Figure 2. Linear correlation between serum prostate-specific antigen (PSA) levels and tumor size. A graph of the correlation between tumor size and serum prostate-specific antigen (PSA) concentration in mice ($n=7$) injected with LNCaP PSA-producing cells indicated a good correlation of these parameters using Spearman's rank test ($\gamma=0.991$, $p<0.05$).

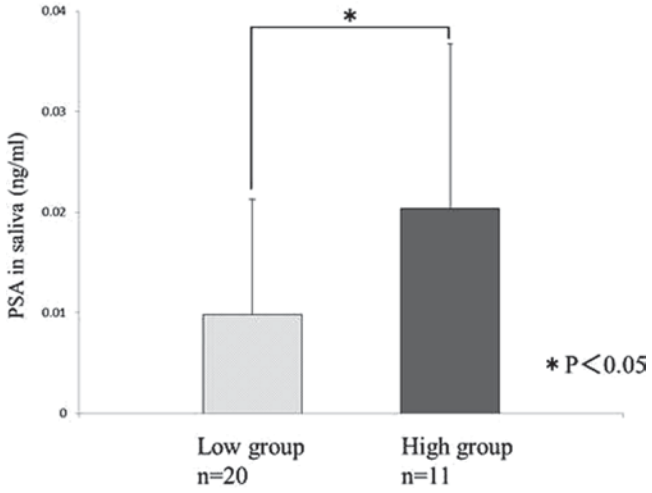

Figure 3. Linear correlation between the prostate-specific antigen (PSA) concentration of submandibular gland tissue and that of serum. A graph of the correlation between the intra-tissue PSA concentration of submandibular gland and serum PSA in the LNCaP mouse model ($n=7$) indicated a weak correlation of these parameters using Spearman's rank test ($\gamma=0.393$, $p<0.05$).

of submandibular gland was dependent on an increase in serum PSA. Lastly, the mean concentration of saliva PSA was 0.381 ± 0.364 ng/mL (range 0.096–0.9 ng/mL) in the LNCaP injection group, but no PSA was detected in the control group (Table 1). However, no correlation between the serum PSA and the saliva PSA was identified using Spearman's rank test.

Human study

RT-PCR analysis of PSA gene expression

Although amplified products corresponding to PSA transcripts were detected in RT-PCR samples of LNCaP, which was used as a positive control for PSA messenger RNA (mRNA), no PSA transcripts were detected in major human salivary glands, including parotid, submandibular, and sublingual glands. The size of the amplified PSA fragment was 289 bp (Figure 4). Signals of β-actin, used as an internal control, were detected in all samples.

PSA concentration in saliva and serum samples

In the high-serum PSA concentration group, the mean PSA values were 22.038 ± 17.611 ng/mL (range 3.837–55.779 ng/mL) in serum and 0.020 ± 0.016 ng/mL (range 0.003–0.056) in saliva. In the low-serum PSA concentration group, the mean values were 0.811 ± 0.678 ng/mL (range 0.043–2.064 ng/mL) in serum and 0.010 ± 0.011 ng/mL (range 0.000–0.039 ng/mL) in saliva. There were significant differences in saliva PSA concentration between the high-serum PSA concentration and low-serum PSA concentration groups ($p<0.05$) (Figure 5). When Spearman's rank test was performed, a significant linear relationship between saliva PSA and serum PSA concentration was determined in the high-serum PSA concentration group ($\gamma=0.910$, $p<0.001$) (Figure 6). There was no correlation between serum PSA and saliva

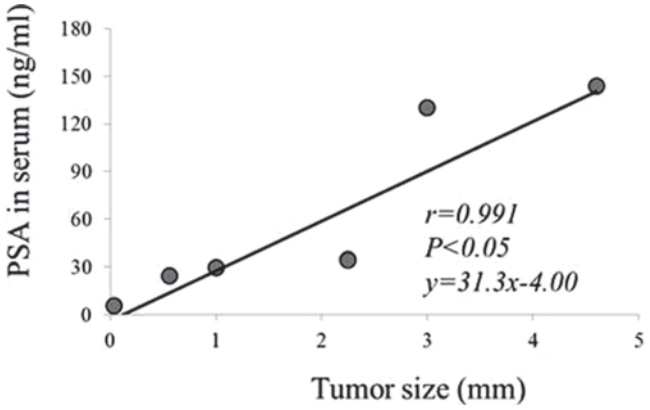

Figure 4. Real-time polymerase chain reaction analysis of prostate-specific antigen (PSA) expression in major human salivary glands. PSA messenger RNA (mRNA) signals were detected in human parotid, submandibular, and sublingual glands. β-actin mRNA expression was used as an internal control. The carcinoma cell line, LNCaP, was used as a positive control.

PSA concentration in the low-serum PSA concentration group was identified. Free PSA was not detected in any samples.

Discussion

SCID mice injected with the human adenocarcinoma cell line LNCaP showed a PSA level that is significantly increased in serum samples, in intra-tissue samples of submandibular gland, and in saliva samples, compared with control SCID mice. LNCaP is well established as a PSA-expressing cell line (Horoszewicz et al. 1980, Horoszewicz et al. 1983). Since mice express little or no PSA, the PSA detected in these samples is considered to have originated from the LNCaP cells (Rittenhous et al. 1998). In addition, there were significant correlations between the PSA level of serum samples and that of intra-tissue samples of the submandibular gland. It is, thus, clear that PSA transits from blood to salivary gland in animals.

PSA mRNA signals were not detected by conventional RT-PCR in major human salivary glands including parotid, submandibular, and sublingual glands, when 25 PCR cycles were used. However, PSA mRNA expression in major salivary glands is controversial. For instance, it has been reported that PSA gene expression is low in salivary glands (Yousef et al. 1999). Ishikawa et al. also reported low-intensity PSA mRNA signals of 2.9% in the salivary gland compared with high-intensity signals of 82.5% in the prostate (Ishikawa et al. 1998). Since these previous reports used a high number of PCR cycles (e.g. 43 or 50) (Ishikawa et al. 1998, Yousef et al. 1999), PSA mRNA signals were probably detected by PCR analysis even when the level of PSA expression was very low. At the protein level, Tazawa et al. reported that prostate showed positive immunoreactivity for the PSA monoclonal antibody ER-PR8, but that human salivary gland failed to demonstrate immunoreactivity to this antibody (Tazawa et al. 1999). In the prostate,

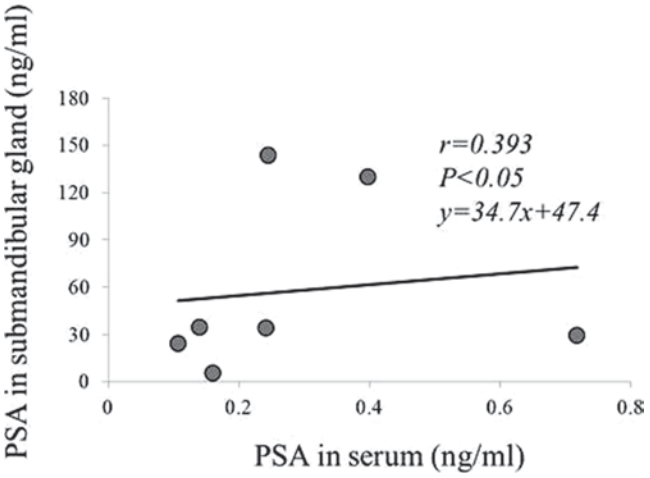

Figure 5. The level of prostate-specific antigen (PSA) in the saliva of the PSA-low and PSA-high patient groups. A significant difference was observed in the PSA level in the saliva of the high-serum PSA concentration and low-serum PSA concentration groups, as determined using Mann–Whitney U-test ($p < 0.05$).

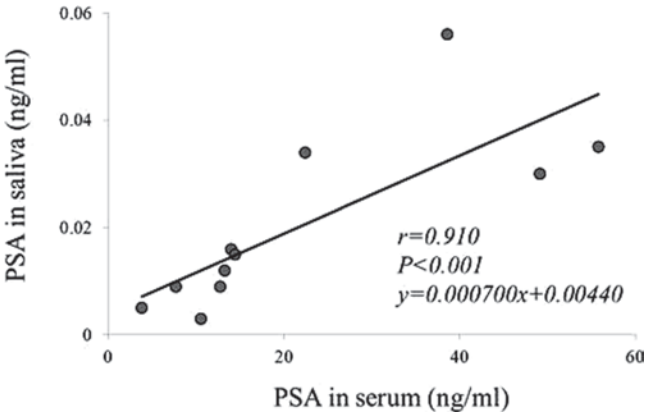

Figure 6. Linear correlation between saliva prostate-specific antigen (PSA) and serum PSA levels in the high- or low-serum PSA concentration groups of patients. The saliva PSA concentration showed a very good correlation with the serum PSA concentration in the high-serum PSA concentration group, as determined using Spearman's rank test ($\gamma = 0.910$, $p < 0.001$).

PSA production is regulated by androgenic steroids, which bind to androgen receptors and upregulate the transcription of PSA (Montgomery et al. 1992). However, sex hormone receptors were not identified in the human salivary gland (Tazawa et al. 1999). In addition, Aksoy et al. suggested that PSA in saliva, rather than being produced in the salivary gland, may reflect serum PSA during the normal menstrual cycle (Aksoy et al. 2002). For these reasons, we believe that human salivary glands produce virtually no PSA protein, even though the PSA gene may be expressed at a low level. The most important conditions for a useful saliva test are that targeted biomarker molecules are little produced by the salivary gland, and that these molecules can transit from blood to acinar cells. We next examined the potential clinical application since saliva PSA adapted to these conditions.

In clinical samples, there were significant differences in the saliva PSA level between the high-serum PSA concentration group and the low-serum PSA concentration group. Saliva PSA concentration showed a very good correlation with serum PSA concentration in the high-serum PSA concentration group using Spearman's rank test. Clinically, measurement of total PSA has served as an excellent indicator of prostate disease when the concentration in serum exceeds 4.0 ng/mL. In subsequent therapy, a total serum PSA level of over 2.5 ng/mL is diagnosed as a sign of recurrence or metastasis (Ito et al. 2000, Saito 2007). In this study, we assayed the saliva PSA level of patients who were divided into high-serum PSA concentration and low-serum PSA concentration groups, based on a serum cut-off value of 2.5 ng/mL. Since recurrence or metastasis was identified in all patients in the high-serum PSA concentration group. A high concentration of saliva PSA during monitoring after therapy may be useful as a method for the detection of recurrence or metastasis.

Ayatollahi et al. reported that there was a significant correlation between the ratio of free/total PSA in the saliva and that of the serum of men with a normal prostate, but that there was no correlation between the total or the free PSA level in saliva and serum (Ayatollahi et al. 2007). Interestingly, it has been reported that the free PSA level of patients with PA was lower than that of patients with benign prostatic hyperplasia. This result is consistent with the fact that free PSA was not detected in the saliva of patients with PA. On the other hand, Aksoy et al. found that there were positive correlations between serum PSA and saliva PSA when the total PSA of healthy women was assayed (Aksoy et al. 2002). However, Breul et al. detected a high concentration of total PSA that ranged between 129 ng/mL and 688 ng/mL in saliva, which did not correlate with the serum concentration (Breul et al. 1993). Therefore, there are discrepancies between the results of reported studies. The different methods used to measure total serum PSA levels may be one of the reasons for these discrepancies in the salivary PSA results. Thus, it is necessary to standardize the saliva PSA measurement of these various methods.

In conclusion, our animal study experiments showed that PSA transits from blood to the salivary gland. In addition, there was a significant correlation between saliva PSA and serum PSA levels in patients with high-serum PSA levels after. Saliva PSA measurements reflected blood PSA levels in patients with recurrent or metastatic PA and may, therefore, be a useful prostate cancer biomarker. Further studies are needed to analyze saliva PSA levels in a large number of patients with PA.

Acknowledgments

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Declaration of interest

The authors report no conflicts of interest.

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